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Substrates and Intermediates in the Enzymatic Reduction of Metmyoglobin in Ground Beef

SUMMARY—Various substrates were tested for their ability to increase the reduction of metmyoglobin by donating electrons to NAD. Intermediates of the glycolytic pathway which proved to be effective were glyceraldehyde-3-phosphate and fructose-1,6-diphosphate. Other substrates, oxidized by NAD-linked dehydrogenases known to be present in meat are α-glycerophosphate, malate and glutamate. These also increased metmyoglobin reduction when added to meat.

The pathway of electron transport from NADH to metmyoglobin is believed to be mainly by way of DT diaphorase (menadione reductase) and quinones, since dicumarol, a specific inhibitor of this enzyme, partially blocked the reaction in meat. Higher levels of quinones did not accelerate the reaction.

INTRODUCTION

THE ACCEPTABILITY of meat is decreased when the brown pigment metmyoglobin is produced. This pigment can be reduced back to purplish myoglobin by enzymes present in meat (Stewart *et al.*, 1965). Watts *et al.* (1966) have shown that the reduction of both metmyoglobin and oxygen in meat is mediated through nicotinamide adenine dinucleotide (NAD) rather than succinic oxidase. Metmyoglobin is reduced only under anaerobic conditions. The nature of the substrates capable of reducing NAD and intermediates between NADH and metmyoglobin were unexplored; these areas will be considered here.

While there are a number of enzyme-substrate systems capable of reducing NAD in living muscle, their activity in post-rigor meat is unexplored. During the storage of intact beef muscle for 2 to 4 weeks, most of the glycolytic enzymes were found to be active (Andrews *et al.*, 1952). Similar studies have been reported in fish muscle (McLeod *et al.*, 1963).

Using histochemical techniques (Ogata *et al.*, 1964; Bodwell *et al.*, 1965), glycolytic, tricarboxylic acid cycle and electron transport chain enzymes have been detected in pork and beef muscles, although activities of some of these enzymes decreased with time (Bodwell *et al.*, 1965). Lack of substrates, rather than loss of enzymes, probably limits reductive capacity of meat after slaughter (Andrews *et al.*, 1952; Bodwell *et al.*, 1965; Watts *et al.*, 1966).

Enzymes may be involved in the transport of electrons from reduced pyridine nucleotide to metmyoglobin. An attempt to isolate a metmyoglobin reductase was not successful (Rossi-Fanelli *et al.*, 1957). However, a cytoplasmic flavoenzyme, DT diaphorase (also known as menadione reductase) was found in meat (Bodwell *et al.*, 1965). This enzyme catalyzes the reduction of various quinones to quinols, which in turn may reduce ferric heme compounds (Ernster *et al.*, 1962; Conover *et al.*, 1962).

The overall metmyoglobin-reducing ability in meat may be influenced by a number of factors such as concentration of NAD in meat, availability of various substrates and of intermediates of electron transport. Addition of NAD to meat was found to increase metmyoglobin reduction (Watts *et al.*, 1966). implying that this pyridine nucleotide could be a limiting factor. The NAD present at the time of slaughter is destroyed by the action of several enzymes which are brought into contact with the nucleotides upon maceration of the tissues (Severin *et al.*, 1963). Whether substrates and electron carriers are also limiting is not known.

A better understanding of the contributions of each of these factors could lead to an improvement in reducing ability by addition of the limiting factor. The purpose of this research is to study the effect of adding various substrates and intermediates on metmyoglobin reduction in meat.

MATERIALS AND METHODS

Chemicals and solutions

Sources of chemicals used in this study were: nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and fructose-1,6-diphosphate (FDP) from Calbiochem; DL-glyceraldehyde-3-phosphate (gly-3-p), DL-α-glycerophosphate, DL-isocitrate, malate, iodoacetate and dicumarol from Sigma Chemical Co.; fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, glutamate and menadione from Nutritional Biochemicals Co.; 1-2-naphthoquinone and p-benzoquinone from Eastman Organic Chemicals; 2-6-dimethylbenzoquinone from K and K Lab., Inc.

The stock solutions were prepared in distilled water except for quinones and dicumarol which were dissolved in propylene glycol or ethyl alcohol. Dilutions were made from the stock solution so that the appropriate amount of the test substance was contained in 1 to 2 ml volume. Controls (without test substance) contained the solvent alone.

DL-glyceraldehyde-3-phosphate solution was prepared from diethylacetal barium salt according to the method described by Sigma Technical Bulletin No. 10 (1961).

Preparation of meat samples

Beef semitendinosus (eye of round) from local packing plants was used. All meat was trimmed of external fat, ground twice and mixed just before each experiment. No attempt was made to adjust the pH of the meat, but solutions to be added were adjusted to the pH of the meat when necessary, so that all variations within a single experiment had the same pH. Fifty-gram portions were

placed in polyethylene bags and stored in the refrigerator at 1 to 5°C until the time of assay (2 to 6 hr).

Metmyoglobin reducing activity was measured as described by Watts *et al.* (1966).

RESULTS AND INTERPRETATION

Addition of substrates capable of supplying hydrogen to NAD

Intermediates of glycolytic pathway. Watts *et al.* (1966) demonstrated that NAD addition to meat increased the reduction of metmyoglobin, but its effectiveness varies considerably in samples of meat from different animals (Saleh, 1967). Varying amounts of suitable substrates may be the limiting factor. Since all of the glycolytic enzymes are still potentially active in post rigor meat, various intermediates of the glycolytic pathway were tested for their ability to increase metmyoglobin reduction.

Glyceraldehyde-3-phosphate. Three experiments on the addition of gly-3-p in the presence of NAD are summarized in Table 1. Metmyoglobin reduction was increased in the treated samples as compared to the controls.

The immediate response to gly-3-p is clearly shown in Fig. 1 when the reduction was measured every 2 min. Whereas 50% reduction of metmyoglobin was obtained in 15 min as a result of gly-3-p addition, only 8% of the pigment was reduced in the control within this time period. The magnitude of gly-3-p response tended to be more pronounced in those experiments in which the reducing ability of the control was low. It is probable that in the more active controls, greater amounts of endogenous substrates were present.

Iodoacetate in low concentration is known to inhibit glyceraldehyde-3-p-dehydrogenase (Cori *et al.*, 1948). The extent of inhibition in pure systems varies from 85 to 95% (Cori *et al.*, 1948; Dixon, 1937).

The effect of iodoacetate on metmyoglobin reduction was investigated in meat containing added gly-3-p. The results (Table 2) show a maximum inhibition of 73%. Other enzyme substrate systems, i.e., glutamate and its associated dehydrogenase (Singer *et al.*, 1954), and malic dehydrogenase (Dixon, 1937) which are not as sensitive to iodoacetate as gly-3-p, may also contribute electrons for the reduction of NAD.

Another possible reason for incomplete inhibition is that in the presence of excess gly-3-p, the enzyme is protected from inhibition by iodoacetate (Segal *et al.*, 1953). The extent of inhibition by iodoacetate was the same in the presence and in the absence of gly-3-p, indicating that this glycolytic intermediate may be an important endogenous substrate in meat.

Table 1. Effect of glyceraldehyde-3-phosphate on metmyoglobin reduction.

Experiment No.	Gly-3-p added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	30
	30	334	....
	60	1170	12
2	0	100	36
	60	650	15
3	0	100	15
	60	175	7
	120	178	5

All samples contained 40 mg % NAD.

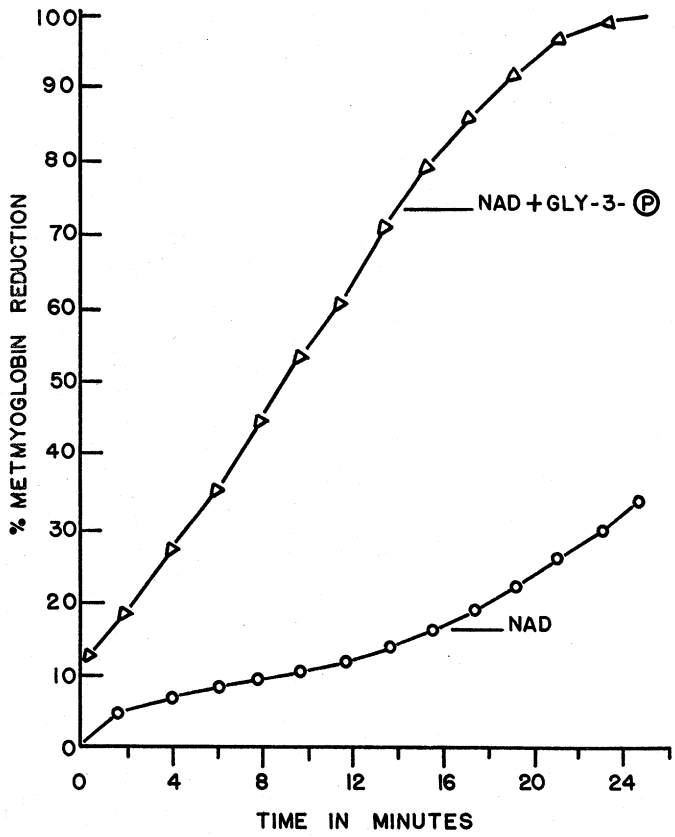


Fig. 1. Effect of gly-3-p on metmyoglobin reduction in ground beef. Both samples contained 40 mg % NAD. Concentration of gly-3-p in the test sample was 60 mg %.

Fructose-1,6-diphosphate (FDP). Gly-3-p can be derived by the hydrolytic cleavage of FDP by the action of aldolase shown to be present in meat (Andrews *et al.*, 1952). FDP was therefore tested for its effect on metmyoglobin reduction. A 2- to 3-fold increase in the rate of reduction of metmyoglobin was noticed in four experiments (Table 3). The results indirectly confirm the finding that aldolase is present in post-rigor meat (Andrews *et al.*, 1952).

When iodoacetate was added to meat along with FDP, a maximum inhibition of 60% in metmyoglobin reduction was observed.

DL-α-glycerophosphate. Another substrate indirectly related to the glycolytic pathway, whose oxidation is dependent on NAD, is α-glycerophosphate. The DL form

Table 2. Effect of iodoacetate on metmyoglobin reduction in the presence of glyceraldehyde-3-phosphate.

Iodoacetate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	5
100	53	15
200	27	28
300	33	29

All samples contained 40 mg % NAD and 60 mg % Gly-3-p.

Table 3. Effect of fructose-1,6-diphosphate on metmyoglobin reduction.

Experiment No.	FDP added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	17
	150	224	5
	300	237	5
2	0	100	28
	300	334	8
3	0	100	18
	300	248	5
4	0	100	20
	300	264	5

All samples contained 40 mg % NAD.

was tested for its effect on metmyoglobin reduction in meat. The enzyme, α-glycerophosphate dehydrogenase, located both in the soluble cytoplasm and in the mitochondria, may act as carrier of the NADH hydrogen across the mitochondrial membrane (Estabrook *et al.*, 1958). This dehydrogenase has been reported to be present in meat (Bodwell *et al.*, 1965). The results of the addition of various amounts of substrate along with NAD are reported in Table 4. Stimulation of metmyoglobin reduction was 1½- to 2-fold.

Glucose and some hexose monophosphates. The results of experiments on the addition to meat of glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate were erratic; metmyoglobin reduction ranged from 25 to 170% of the control. No consistent increase in reduction was obtained with any of the compounds.

The observation that FDP addition resulted in stimulation of metmyoglobin reduction, whereas none of the monophosphate sugars gave such an effect, demonstrates the importance of the second phosphorylation. ATP is necessary for the conversion of monophosphorylated sugars to sugar diphosphates. Lack of ATP in post-rigor meat may limit this reaction.

Although lactate is present in large amounts in post-rigor meat and its oxidation is NAD dependent, the equilibrium of the lactic dehydrogenase reaction favors the oxidation rather than the reduction of NAD. Lactate was, therefore, not used as a test substance.

Some tricarboxylic acid cycle intermediates

Isocitrate. Early in this study it was observed that the addition of NADP to meat either had no effect or slightly

Table 4. Effect of DL-α-glycerophosphate on metmyoglobin reduction.

Experiment No.	DL-α-glycerophosphate added mg %	Metmyoglobin reduction in 10 min % of control	Time for 50% reduction (min)
1	0	100	15
	40	130	11
	200	197	6
	400	186	6
2	0	100	13
	200	156	8
	400	158	8

All samples contained 40 mg % NAD.

Table 5. Effect of DL-isocitrate on metmyoglobin reduction in presence of NADP.

DL-isocitrate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	51
20	136	41
40	236	26
60	264	23

All samples contained 20 mg % NADP.

inhibited metmyoglobin reduction. However, isocitrate addition, along with NADP, stimulated reduction as compared to NADP controls (Table 5). The presence of isocitrate dehydrogenase has been demonstrated in post-rigor meat (Bodwell *et al.*, 1965). From these results it appears that the ineffectiveness of NADP additions to meat may be ascribed to the lack of NADP-linked substrates.

Isocitrate addition, along with NAD to meat, inhibited metmyoglobin reduction. The reason for this inhibitory effect is not clear.

L-malate. Another substrate of the citric acid cycle, the oxidation of which proceeds via NAD, is malate. When L-malate was added to meat in the presence of NAD, it increased the reduction of metmyoglobin by a factor of 1½ to 2 (Table 6). This is presumptive evidence for the presence of malic dehydrogenase in post-rigor meat.

L-glutamate

Glutamate has been shown to be present as part of the free amino acid pool in meat (Gardner *et al.*, 1966). In addition, it is an inexpensive substrate, and is used widely in foods to enhance flavor. The enzyme catalyzing its oxidation, glutamic dehydrogenase, has also been reported in meat although its activity was somewhat low (Ogata *et al.*, 1964; Bodwell *et al.*, 1965).

The results of five experiments using different concentrations of L-glutamate are shown in Table 7. All five experiments were carried out under anaerobic conditions and additional NAD had been added to the meat samples. Results ranged from no effect in one experiment to marked stimulation.

Since L-glutamate gave promising results, it was tested further under both aerobic and anaerobic conditions and in the presence and absence of added NAD. The study under aerobic conditions was prompted by the fact that, in practice, meat is generally marketed in packaging material permeable to oxygen. The results are shown in Table 8. With all experimental variations, glutamate enhanced the reduction of metmyoglobin. The acceleration due to glutamate was greatest under aerobic conditions, without

Table 6. Effect of L-malate on metmyoglobin reduction.

L-malate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	24
140	164	16
280	193	14

All samples contained 40 mg % NAD.

Table 7. Effect of L-glutamate on metmyoglobin reduction.

Experiment No.	L-glutamate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	12
	20	191	8
	200	230	5
	400	200	8
2	0	100	13
	200	133	10
	400	197	7
3	0	100	12
	200	136	10
4	0	100	16
	200	113	14
5	0	100	14
	200	102	12

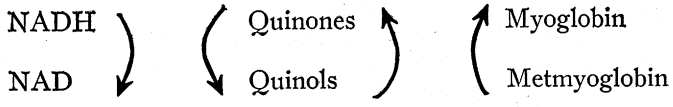
All samples contained 40 mg % NAD.

added NAD. Under these conditions the metmyoglobin reducing ability of the control itself was low.

In two additional experiments, meat treated with glutamate was stored for some days under both aerobic and anaerobic conditions and its reducing activity compared with untreated controls. The metmyoglobin-reducing activity of the glutamate-treated samples remained high over the storage period, whereas reduction in the controls decreased steadily. However, neither spectrophotometrically determined metmyoglobin nor subjective color ratings were significantly different in stored treated samples versus controls.

Role of quinones as possible intermediates

Quinones have been reported to mediate metmyoglobin reduction by NADH or NADPH in pure systems (Rossi-Fanelli *et al.*, 1957). DT diaphorase, a cytoplasmic enzyme, is present in meat (Bodwell *et al.*, 1965) and may be involved in metmyoglobin reduction. The purified enzyme has been shown to catalyze the oxidation of both NADH and NADPH with various quinones acting as electron acceptors (Ernster *et al.*, 1962). The role of quinones in the electron flow from NADH to metmyoglobin can be schematically presented as follows:



Various quinones were tested to determine whether insufficient quinone might be a limiting factor in metmyoglobin reduction in meat. Menadione, (2-methyl-1,4-naphthoquinone), 1,2-naphthoquinone, p-penzoquinone, and 2,6-dimethylbenzoquinone were tried. They either had no effect or somewhat inhibited reducing activity. During the mixing of some of the quinones (particularly naphthoquinone) with the ground meat, a rapid oxidation of meat pigments to metmyoglobin (brown color) was observed before the actual addition of the oxidizing agent (ferricyanide) to meat.

It is likely that there already are enough quinones present in meat to mediate the transport of electrons from

Table 8. Effect of L-glutamate on metmyogobin reduction under aerobic and anaerobic conditions.

Treatment	Time for 50% metmyoglobin reduction (min)	Metmyoglobin reduction % of control in 15 min	25 min
Anaerobic			
NAD-control	14	100	....
NAD + L-glutamate	11	130	....
Control—(no NAD)	50	100	....
Control + L-glutamate	28	342	....
Aerobic			
NAD-control	31	....	100
NAD-L-glutamate	17	....	220
Control—(no NAD)	50[1]	....	100
Control + L-glutamate	29[1]	....	600

[1] Time for 20% metmyoglobin reduction.
Concentrations used: NAD, 40 mg %; L-glutamate, 400 mg %.

NADH to metmyoglobin. Additional amounts might be expected to have an adverse effect on metmyoglobin reduction. Harley *et al.* (1962a,b) found the concentration of menadione to be critical in the enzymatic reduction of methemoglobin. Higher concentrations not only oxidize oxyhemoglobin to methemoglobin but also inactivate certain of the cellular enzymes concerned with the reduction of methemoglobin. Magos (1964) has shown that quinones not only oxidize ferrous hemes but also bring about the formation of globin hemochromogens.

The effect of dicumarol on metmyoglobin reduction.

DT diaphorase has been reported to be strongly inhibited by dicumarol (Ernster *et al.*, 1962). Moreover, dicumarol seems to be highly specific for this enzyme. Other diaphorase activities found in rat liver were not appreciably affected by even a 1,000-fold excess of the concentration needed for a complete inhibition of the DT diaphorase.

The results of two experiments on dicumarol addition to ground meat are shown in Table 9. The concentrations of dicumarol used ranged from 0.2 to 20 mg %. Inhibition varied from 0 to 63%. The dicumarol was difficult to dissolve; even at 0.2 mg/ml a true solution could not be obtained. The actual amount in solution at the higher concentration is not known.

The partial inhibition indicates that DT diaphorase plays a role as an intermediate in metmyoglobin reduction. The fact that inhibition was not complete may mean either that

Table 9. Effect of dicumarol on metmyoglobin reduction.

Experiment No.	Dicumarol added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	17
	0.2	100	17
	0.4	66	22
	1.0	80	21
2	0	100	14
	0.2	100	14
	0.4	44	20
	1.0	62	19
	20	37	20

All samples contained 40 mg % NAD.

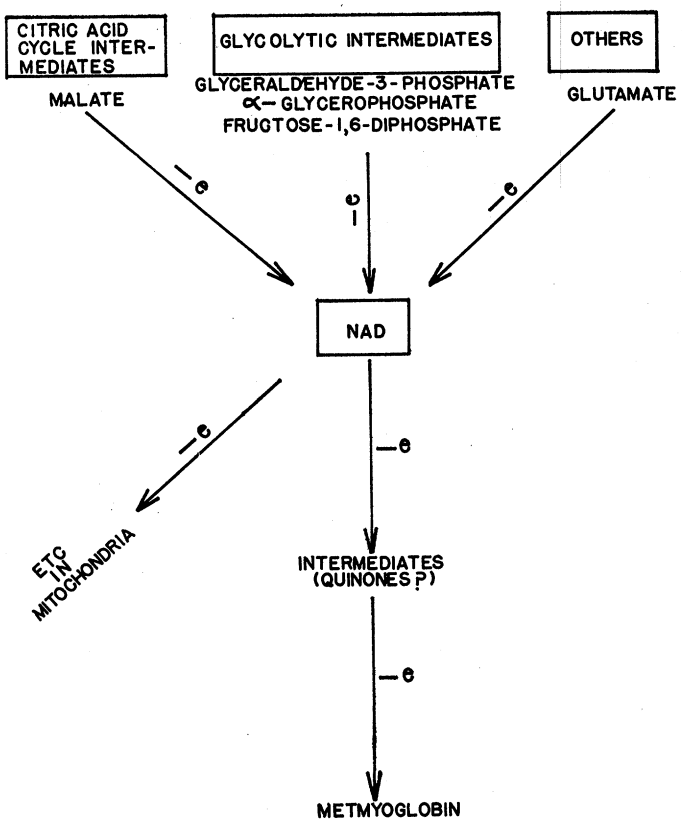


Fig. 2. Hypothetical scheme for the role of substrates and intermediates in metmyoglobin reduction in meat. ETC = electron transport chain.

DT diaphorase is not completely inhibited or that there is an additional pathway for the transport of electrons from NADH to metmyoglobin. Rossi-Fanelli *et al.* (1957) showed that the non-enzymatic reduction of metmyoglobin by NADH occurs at a measurable rate in the presence of quinones.

DISCUSSION

FROM THE RESULTS obtained in the present investigation, a number of substrates, any one of which may be present in trace amounts in meat, may be utilized to supply electrons to NAD and metmyoglobin according to the following scheme (Fig. 2). This work does not shed light on the actual endogenous substrates responsible for reducing activity in meat. However, it does demonstrate that, in meat, the rate of reduction of NAD controls the over-all rate of reduction of metmyoglobin. The step or steps between NADH and metmyoglobin apparently proceed rapidly, once NAD is reduced.

This work also demonstrates that the reducing activity of most samples of meat can be increased by the addition of appropriate substrates. From a practical standpoint, of the substrates used, only monosodium glutamate is cheap enough to be seriously considered.

Insufficient quantities of NAD also limit reducing activity of most samples of meat. Again NAD is far too expensive to be considered as an additive to meat except for experimental purposes. However, practical treatments which will protect the NAD normally present in meat have been investigated and will be described in the near future.

The larger question of the possible benefits to be derived from increasing the reducing activity of meats is still unclear. Hutchins *et al.* (1967) found a significant but not very high correlation between the reducing activity of meat from different animals and their tendency to resist browning (pigment oxidation) during refrigerated storage. However, in the present study, when the reducing activity of a single batch of meat was increased by the addition of glutamate, no significant improvement in appearance or decrease in metmyoglobin was noted in the treated samples after refrigerated storage.

The problem is complicated by the fact that autoxidation of myoglobin is much more rapid at low oxygen tensions than in air. Thus, the partial removal of oxygen from packaged meat by enzymatic activity could accelerate autoxidation. A systematic study of the relation of reducing activity to meat color under a variety of storage conditions is needed.

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